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(57) Abstract			
An improved process for the preparation of adipoyl cephalosporins via enzymatic ring expansion of adipoyl-6-aminopenicillanic acid, using a <i>Penicillium chrysogenum</i> transformant strain expressing modified expandase enzyme.			
U.S. Patent Application No. 10/719,237 Attorney Docket No. 6653-020-999 Reference AM			

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IMPROVED PROCESS FOR THE PRODUCTION OF  
ADIPOYL CEPHALOSPORINS

Field of the invention and brief description of the prior  
5 art

The present invention concerns a biosynthetic process for preparation and recovery of adipoyl cephalosporins (5-carboxypentanoyl cephalosporins). Adipoyl-7-aminocephalo-  
10 sporins include adipoyl-7-aminodesacetoxycephalosporanic acid, adipoyl-7-aminodesacetylcephalosporanic acid and adipoyl-7-aminocephalosporanic acid. The 7-aminocephalo-  
sporines which can be obtained after deacylation of the adipoyl cephalosporins, 7-aminodesacetoxycephalosporanic  
15 acid (7-ADCA), 7-aminodesacetylcephalosporanic acid or 7-aminocephalosporanic acid (7ACA) respectively, are key intermediates used in the preparation of semi-synthetic cephalosporins (SSC's).

20  $\beta$ -Lactam antibiotics constitute the most important group of antibiotic compounds, with a long history of clinical use. Among this group, the prominent ones are the penicillins and cephalosporins. These compounds are naturally produced by the filamentous fungi *Penicillium*  
25 *chrysogenum* and *Acremonium chrysogenum*, respectively.

As a result of classical strain improvement techniques, the production levels of the antibiotics in *Penicillium chrysogenum* and *Acremonium chrysogenum* have increased dramatically over the past decades. With the  
30 increasing knowledge of the biosynthetic pathways leading to penicillins and cephalosporins, and the advent of recombinant DNA technology, new tools for the improvement of production strains and for the *in vivo* derivatization of the compounds have become available.

35 Most enzymes involved in  $\beta$ -lactam biosynthesis have been identified and their corresponding genes been cloned, as can be found in Ingolia and Queener, Med. Res. Rev. 9

(1989), 245-264 (biosynthesis route and enzymes), and Aharonowitz, Cohen, and Martin, Ann. Rev. Microbiol. 46 (1992), 461-495 (gene cloning).

The first two steps in the biosynthesis of penicillin in *P. chrysogenum* are the condensation of the three amino acids L-5-amino-5-carboxypentanoic acid (L- $\alpha$ -aminoadipic acid) (A), L-cysteine (C) and L-valine (V) into the tripeptide LLD-ACV, followed by cyclization of this tripeptide to form isopenicillin N. This compound contains the typical  $\beta$ -lactam structure.

The third step involves the exchange of the hydrophilic side chain of L-5-amino-5-carboxypentanoic acid by a hydrophobic side chain by the action of the enzyme acyltransferase (AT). The enzymatic exchange reaction mediated by AT takes place inside a cellular organelle, the microbody, as has been described in EP-A-0448180.

Cephalosporins are much more expensive than penicillins. One reason is that some cephalosporins (e.g. cephalixin) are made from penicillins by a number of chemical conversions. Another reason is that, so far, only cephalosporins with a D-5-aminoadipoyl side chain could be fermented. Cephalosporin C, by far the most important starting material in this respect, is very soluble in water at any pH, thus implying lengthy and costly isolation processes using cumbersome and expensive column technology. Cephalosporin C obtained in this way has to be converted into therapeutically used cephalosporins by a number of chemical and enzymatic conversions.

The methods currently favoured in industry to prepare the intermediate 7-ADCA involve complex chemical steps leading to the expansion and derivatization of penicillin G. One of the necessary chemical steps to produce 7-ADCA involves the expansion of the characteristic 5-membered ring structure of penicillins to the typical 6-membered ring structure of cephalosporins (see for instance US 4,003,894). This complex chemical processing is both expensive and noxious to the environment.

Consequently, there is a great desire to replace such chemical processes with enzymatical reactions such as

enzymatic catalysis, preferably during fermentation. A key to the replacement of the chemical expansion process by a biological process is the central enzyme in the cephalosporin biosynthetic pathway, desacetoxyccephalosporin C synthase (DAOCS), or expandase.

The expandase enzyme from the bacterium *Streptomyces clavuligerus* has been well characterized (EP-A-0366354) both biochemically and functionally, as has its corresponding gene. Both physical maps of the *cefE* gene (EP-A-0341892),  
10 DNA sequence and transformation studies in *P. chrysogenum* with *cefE* have been described. When introduced into *P. chrysogenum*, it can convert the penicillin ring structure into the cephalosporin ring structure, as described in Cantwell et al., Proc. R. Soc. Lond. B. 248 (1992), 283-289.

15 Other sources for a ring expansion enzyme are the bacteria *Nocardia lactamdurans* (formerly *Streptomyces lactamdurans*) and *Lysobacter lactamgenus*. Both the biochemical properties of the enzyme and the DNA sequence of the gene have been described for *Nocardia lactamdurans*  
20 (Cortés et al., J. Gen. Microbiol. 133 (1987), 3165-3174; and Coque et al., Mol. Gen. Genet. 236 (1993), 453-458, respectively). For *Lysobacter lactamdurans* the gene cluster involved in cephalosporin biosynthesis was sequenced and sequences of several key enzymes were deposited to the EMBL  
25 Data Library (Kimura et al., October 1990, entry code EMBL:X56660).

It has recently been found that the expandase enzyme is capable of expanding penicillins with particular side chains to the corresponding 7-ADCA derivative. This feature  
30 of the expandase has been exploited in the technology as disclosed in EP-A-0532341, WO95/04148 and WO95/04149. In these disclosures the conventional chemical conversion of penicillin G to 7-ADCA has been replaced by the *in vivo* conversion of certain 6-aminopenicillanic acid (6-APA)  
35 derivatives in recombinant *Penicillium chrysogenum* strains containing an expandase gene.

In EP-A-0532341 the application of an adipate (5-carboxypentanoate) feedstock has been disclosed. The incorporation of this substrate leads to a penicillin

derivative with an adipoyl side chain, viz. adipoyl-6-APA. This incorporation is due to the fact that the acyltransferase has a proven wide substrate specificity (Behrens et al., J. Biol. Chem. 175 (1948), 751-809; Cole, Process. Biochem. 1 (1966), 334-338; Ballio et al., Nature 185 (1960), 97-99).

More particularly, EP-A-0532341 teaches the *in vivo* use of the expandase enzyme in *P. chrysogenum*, in combination with a adipoyl side chain as a feedstock, which is used as a substrate for the acyltransferase enzyme in *P. chrysogenum*. This leads to the formation of adipoyl-6-APA, which is converted by an expandase enzyme introduced into the *P. chrysogenum* strain to yield adipoyl-7-ADCA. Finally, the removal of the 5-adipoyl side chain is suggested, yielding 7-ADCA as a final product. The patent application EP-A-0540210 describes a similar process for the preparation of 7-ACA, including the extra steps of converting the 3-methyl side chain of ADCA into the 3-acetoxymethyl side chain of ACA.

In WO95/04148 and WO95/04149 it has been disclosed that 3'-carboxymethylthiopropionic acid and 3,3'-thiodipropionic acid, respectively were found to be substrates for the expandase, yielding respectively 2-(carboxyethylthio)acetyl-7-ADCA and a mixture of 3-(carboxymethylthio)propionyl-7-ADCA and 2-(carboxyethylthio)acetyl-7-ADCA. In addition a process was described for the recovery of these cephalosporins from the fermentation broth and the subsequent removal of the side chains by an enzymatic process.

The alternative side chains which are provided above allow for the production of anionic cephalosporins instead of the conventional zwitterionic cephalosporins such as cefC. This allows for a more simple isolation procedure. In addition these side chains can be removed by an enzymatic process. As a consequence these alternative side chains can be regarded as ideal protecting groups for the 7 amino position of the cephalosporin. Due to their beneficial properties mentioned above, cephalosporins with these alternative side chains form a useful starting point for



chemical synthesis where it is required to protect the 7 amino position of the cephalosporin ring.

The observation that substantial quantities of desacetoxyccephalosporin C (DAOC) can be formed by non-precursed *P. chrysogenum* transformants expressing expandase implies the presence of significant amounts of penicillin N, the natural substrate for expandase, in *P. chrysogenum* (Alvi et al. (1995), J. Antibiot. 48, p338-340). As a consequence, with a adipoyl side chain as feedstock, in the *P. chrysogenum* transformants which express expandase activity, penicillin N competes with adipoyl-6-APA for ring expansion resulting in substantial formation of  $\alpha$ -(D) aminoadipoyl-7ADCA (DAOC) at the expense of desired product adipoyl-7-ADCA. In addition to the accumulation of  $\alpha$ -(D) aminoadipoyl-7ADCA, part of the intermediate adipoyl-6-APA is excreted before ring expansion by expandase can occur. As a consequence of producing these by-products additional precautions have to be taken in order to remove these by-products during the recovery of adipoyl-7ADCA. Apart from recovery problems the production of these by-products is a significant waste of  $\beta$ -lactam producing capacity of the strains which ultimately limits the final yield of adipoyl-7ADCA. Redirecting of this  $\beta$ -lactam by-product waste stream into the main adipoyl-7-ADCA synthesis route would benefit the final fermentation yield of adipoyl cephalosporin with regard to yield as well as with regard to the quality of the product.

Recently, the structure of the isopenicillin N synthetase (IPNS) enzyme of *A. nidulans* (aIPNS) has been determined (Roach (1995), Nature, 375, p700-704). IPNS and expandase belong to the same family of oxidase enzymes. They share biochemical characteristics and, on the basis of sequence homologies, it has been proposed that structural similarities exist between the two enzymes (Roach et al., supra; Cooper (1993), Bioorganic Med. Chem. 1, p1-17).

The mechanism of IPNS activity has been described in several reports (see for example: Blackburn et al. (1995), Biochemistry 34, p7548-7562). It is proposed, from an analysis of the chemistry catalysed by IPNS, that the

cysteiny l thiol group of ACV must bind to the ferrous ion at the active site in the enzyme-substrate complex. Given this implicit attachment point between the substrate and the enzyme a large number of conformationally distinct binding modes can be distinguished given the crystallographically determined constraints of the active site. It is therefore not obvious how ACV binds to aIPNS and, by inference, the mode of binding of penicillin N to expandase is even less apparent.

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### Brief description of the figures

Figure 1: Sequence alignment of Isopenicillin N synthetases (IPN synthetases) with expandases (desacetoxy-  
15 cephalosporin C synthases or DAOCS) and cephalosporin 3'-hydroxylases (desacetylcephalosporin C synthase or DACS). Listed are IPN synthetase *Aspergillus nidulans*, *Streptomyces clavuligerus*, *Streptomyces anulatus*, *Streptomyces lactamdurans*, *Flavobacterium* sp. (strain SC 12154),  
20 *Streptomyces griseus* (strain SC 12154), *Lysobacter lactamgenus*, *Streptomyces jumonjinensis*, *Streptomyces cattleya*, DAOCS of *Streptomyces clavuligerus*, DACS of *Streptomyces clavuligerus*, DACS of *Streptomyces lactamdurans*, DAOCS/DACS of *Cephalosporium acremonium*, DACS  
25 of *Lysobacter lactamgenus* (strain YK90), DACS of *Lysobacter lactamgenus* (strain YK90).

Figure 2: Schematic representation of plasmid pZEx.

Figure 3: Schematic representation of plasmid pZExD96N.

Figure 4: Schematic representation of plasmid pZExD96Q.

30 Figure 5: Schematic representation of plasmid pZExD96M.

Figure 6: Schematic representation of plasmid pZExD96K.

Figure 7: Schematic representation of plasmid pZExD96H.

### Summary of the invention

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The present invention provides a more efficient process for the preparation and recovery of adipoyl cephalosporins by:

- a) transforming a *Penicillium chrysogenum* strain with an expandase gene encoding a modified expandase enzyme, under the transcriptional and translational regulation of fungal expression signals;
- 5 b) fermenting said strain in a culture medium and adding to said culture medium adipic acid or a salt or ester thereof suitable to yield adipoyl-6-APA, which is expanded to form adipoyl-7-ADCA;
- c) recovering the adipoyl-7-ADCA from the fermentation  
10 broth;
- d) deacylating adipoyl-7-ADCA; and
- e) recovering the crystalline 7-ADCA.

In particular the process exhibits a better efficiency because the production of adipoyl-7ADCA is improved relative  
15 to production of the main by-products  $\alpha$ -D-aminoadipoyl-7-ADCA (DAOC) and adipoyl-6-APA.

Preferably, adipoyl-7-ADCA is recovered from the fermentation broth by extracting the broth filtrate with an organic solvent immiscible with water at a pH of lower than  
20 about 4.5 and back-extracting the same with water at a pH between 4 and 10.

Moreover, the DNA encoding modified expandase and a recombinant DNA vector comprising the same, functionally linked to the transcriptional and translational control  
25 elements of a fungal gene, for instance *Aspergillus nidulans* *gpdA* gene, and the *P. chrysogenum* *pcbC* gene and host cells transformed with the same, are provided.

#### Detailed description of the invention

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The present invention concerns the use of functional gene constructs encoding modified expandase enzyme in *P. chrysogenum* for the *in vivo* expansion of the adipoyl-6-APA to form the adipic acid derivative of a key intermediate in  
35 the cephalosporin biosynthesis, 7-aminodesacetoxycephalosporanic acid, or 7-ADCA. This derivative has a chemical composition so as to allow efficient solvent extraction, thus providing an economically attractive recovery process.

Modification of the expandase gene is directed at producing expandase mutants which best expand adipoyl-6-APA in *in vitro* and/or *in vivo* context where other penicillins such as penicillin N and isopenicillin N can act as competing substrates. This is an essential feature of the invention given the observation of significant amounts of penicillin N being produced by *P. chrysogenum* and the knowledge that penicillin N is a significantly better substrate than adipoyl-6-APA for the wildtype expandase. By transforming *P. chrysogenum* with such targeted mutants of expandase, novel *P. chrysogenum* strains can be obtained which have an improved capacity for the production of adipoyl-7-ADCA.

The ring expansion of adipoyl-6-APA is a key step in the production of adipoyl cephalosporins. In *P. chrysogenum* strains which are only transformed with the expandase gene, adipoyl-7-ADCA is the end product of the fermentation. When in addition *P. chrysogenum* expresses deacetylcephalosporin C synthase (DACS; the *cefF* gene in *Streptomyces*, the *cefEF* gene in *Acremonium*) as well then adipoyl-7-desacetylcephalosporanic acid is the end product. When finally also desacetylcephalosporin C acetyltransferase (the *cefG* gene) is expressed then adipoyl-7-ACA is produced. More efficient production of adipoyl-7-ADCA will also improve production of the other adipoyl-cephalosporins.

Transformation of *P. chrysogenum* can, in principle, be achieved by different means of DNA delivery, like PEG-Ca mediated protoplast uptake, electroporation or particle gun techniques, and selection of transformants. See for example Van den Hondel en Punt, Gene and Transfer and Vector Development for Filamentous Fungi, in: Applied Molecular Genetics of Fungi (Peberdy, Laten, Ogden, Bennett, eds.), Cambridge University Press (1991). The application of dominant and non-dominant selection markers has been described (Van den Hondel, supra). Selection markers of both homologous (*P. chrysogenum* derived) and heterologous (non-*P. chrysogenum* derived) origin have been described (Gouka et al., J. Biotechnol. 20 (1991), 189-200).

The application of the different transformant selection markers, homologous or heterologous, in the presence or absence of vector sequences, physically linked or not to the non-selectable DNA, in the selection of transformants are well known.

The ring-expansion reaction, mediated by the modified expandase enzyme is introduced into and expressed in this way in *P. chrysogenum*, for instance in strain Wisconsin 54-1255 (deposited at ATCC under accession number 28089). Other strains of *P. chrysogenum*, including mutants of strain Wisconsin 54-1255, having an improved beta-lactam yield, are also suitable.

Furthermore, the modified *cefE* gene is placed under the transcriptional and translational control of fungal (be they filamentous or not) gene control elements. Those elements can be obtained from cloned fungal genes like the *P. chrysogenum* IPNS gene, the  $\beta$  tubulin gene, the *Aspergillus nidulans* *gpdA* gene, or the *Aspergillus niger* *glcA* gene.

In summary, the present invention teaches how the activity of a modified expandase enzyme expressed by a mutated gene which is introduced into *P. chrysogenum*, can be used to improve the yield of adipoyl cephalosporins resulting from the *in vivo* ring expansion of adipoyl-6-APA.

In accordance with the present invention the  $\beta$ -lactam intermediate adipoyl-7-ADCA is produced in *P. chrysogenum* by adding adipic acid or a salt or an ester thereof to the medium. Suitable salts are for instance those of sodium or potassium. Adipoyl-7-ADCA is efficiently recovered from the medium through a simple solvent extraction, for instance, as follows:

The broth is filtered and an organic solvent immiscible with water is added to the filtrate. The pH is adjusted in order to extract the cephalosporin from the aqueous layer. The pH range has to be lower than 4.5; preferably between 4 and 1, more preferably between 2 and 1. In this way the cephalosporin is separated from many other impurities present in the fermentation broth. Preferably a small volume of organic solvent is used, giving a more concentrated solution of the cephalosporin, so achieving

reduction of the volumetric flow rates. A second possibility is whole broth extraction at a pH of 4 or lower. Preferably the broth is extracted between 4 and 1 with an organic solvent immiscible with water.

Any solvent that does not interfere with the cephalosporin molecule can be used. Suitable solvents are, for instance, butyl acetate, ethyl acetate, methyl isobutyl ketone, alcohols like butanol etc.. Preferably 1-butanol or isobutanol are used.

Hereafter the cephalosporin is back extracted with water at a pH between 4 and 10, preferably between 6 and 9. Again the final volume can be reduced. The recovery can be carried out at temperatures between 0 and 50°C, and preferably at ambient temperatures.

The aqueous cephalosporin solution thus obtained is treated with a suitable enzyme in order to remove the adipoyl side chain and obtain the desired 7-ADCA.

Preferably, an immobilized enzyme is used, in order to be able to use the enzyme repeatedly. The methodology for the preparation of such particles and the immobilization of the enzymes have been described extensively in EP-A-0222462. The pH of the aqueous solution has a value of, for example pH 4 to pH 9, at which the degradation reaction of cephalosporin is minimized and the desired conversion with the enzyme is optimized. Thus, the enzyme is added to the aqueous cephalosporin solution while maintaining the pH at the appropriate level by, for instance, adding an inorganic base, such as a potassium hydroxide solution, or applying a cation exchange resin. When the reaction is completed the immobilized enzyme is removed by filtration. Another possibility is the application of the immobilized enzyme in a fixed or fluidized bed column, or using the enzyme in solution and removing the products by membrane filtration. Subsequently, the reaction mixture is acidified in the presence of an organic solvent immiscible with water.

Suitable enzymes are, for instance, derived from a *Pseudomonas* SY77 microorganism having a mutation in one or more of the positions 62, 177, 178 and 179. Also enzymes from other *Pseudomonas* microorganisms, preferably

*Pseudomonas* SE83, optionally having a mutation in one or more of the positions corresponding to the 62, 177, 178 and 179 positions in *Pseudomonas* SY77, may be used.

After adjusting the pH to about 0.1 to 1.5, the layers are separated and the pH of the aqueous layer is adjusted between 2 and 5, more preferably between 3 and 4. The crystalline 7-ADCA is then filtered off.

The deacylation can also be carried out chemically as known in the prior art, for instance, via the formation of an iminochloride side chain, by adding phosphorus pentachloride at a temperature of lower than 10°C and subsequently isobutanol at ambient temperatures or lower.

The following examples are offered by way of illustration and not by way of limitation. The overall approach entails i) identification of residues of expandase involved in substrate specificity, ii) construction of mutant expandase proteins, iii) subcloning of mutant expandase genes in *P. chrysogenum* expression vectors and expression of the mutant expandase in *P. chrysogenum*, iv) determination of the adipoyl-7-ADCA production versus production of  $\alpha$ -D-aminoadipoyl-7-ADCA and adipoyl-6-APA.

In a similar way as has been described for the adipoyl side chain a person skilled in the art may also adapt the expandase enzyme towards the processes as have been disclosed in WO95/04148 and WO95/04149 which use 3'-carboxymethylthiopropionic acid and 3,3'-thiodipropionic acid as side chains, yielding 2-(carboxyethylthio)acetyl-7-ADCA and a mixture of 3-(carboxymethylthio)propionyl-7-ADCA and 2-(carboxyethylthio)acetyl-7-ADCA respectively.

#### Example 1

Identification of residues involved in the binding of the  $\alpha$ -amino group of the adipoyl side chain.

Central to the invention is the proposal that, in the case of aIPNS, upon ACV binding, the L- $\alpha$ -aminoadipoyl side chain of ACV displaces the C-terminal tail of the enzyme (glutamine 330, threonine 331 and a number of preceding residues) by virtue of the similarity between the L- $\alpha$ -

aminoadipoyl side chain of ACV and the C-terminal dipeptide in steric and electronic terms. Comparison of the C-terminal tail and ACV reveals the similarity between the L- $\alpha$ -aminoadipoyl side-chain of ACV the glutaminy-threonine end of the tail; specifically the carboxylates in both cases are functionally homologous. The relatedness of expandase to aIPNS suggests that the D- $\alpha$ -aminoadipoyl side chain of the substrate penicillin N binds in a similar fashion to expandase as does the L- $\alpha$ -aminoadipoyl side chain of ACV to aIPNS. At the heart of the invention is the proposal that the D- $\alpha$ -aminoaminoadipoyl side chain of penicillin N will be bound by amino acid residues of expandase that are homologous to the amino acid residues of aIPNS involved in binding the L- $\alpha$ -aminoadipoyl side chain of ACV.

We propose the  $\alpha$ -carboxyl group of the  $\alpha$ -aminoadipoyl moiety as the major determinant in the substrate for binding to both aIPNS and expandase. As a consequence it is likely that the binding site for this carboxylgroup is conserved between aIPNS and expandase. Expandase is strictly selective for the D-enantiomer of the  $\alpha$ -aminoadipoyl moiety, resulting in the exclusive expansion of Penicillin N. The same preference for the D-enantiomer holds for the desacetylcephalosporin C synthases (DACS) which show a high degree of homology with the expandases. In the cyclization of the tripeptide LLD-ACV the L-enantiomer of  $\alpha$ -aminoadipoyl moiety is converted by aIPNS. However aIPNS is not very selective and can also convert an ACV tripeptide which contains the D-enantiomer of the  $\alpha$ -aminoadipoyl side chain. As a consequence we propose that the binding site for the  $\alpha$ -carboxylgroup of the  $\alpha$ -aminoadipoyl side chain is conserved between the IPNS family and the expandase/hydroxylase family. The binding site of the  $\alpha$ -amino group is expected to be conserved quite strictly within the group of expandases/hydroxylases, but less well between the IPN synthases and the expandases/hydroxylases.

In general positively charged aminogroups in the substrate are often accommodated by negatively charged residues in the protein. Therefore we aligned the expandases/hydroxylases (DAOCS/DACS) which are known at



present (Figure 1). There are 12 positions which exhibit complete conservation of a negative charge. Comparing these positions with the corresponding positions in the IPN synthetases reveals that only 5 of these positions also contain a strictly conserved negative charge in IPN synthetases. From the other positions which do not maintain a negative charge in IPN synthetases only the position in expandase, which corresponds to Asparagine 109 in aIPNS, is close enough to the proposed binding site of the  $\alpha$ -aminoadipoyl side chain to contribute to the specific binding of the  $\alpha$ -amino group. Deletion of the negative charge in the expandases at the position corresponding to position 109 in aIPNS will decrease the specificity for the  $\alpha$ -amino group of the  $\alpha$ -D-aminoadipoyl side chain (penicillin N) relative to the adipoyl moiety (adipoyl-6-APA) which does not contain the amino group. Deletion of the negative charge in expandase can be performed by site-directed mutagenesis. Substitution of the negative charge at the position corresponding with aIPNS 109 will alter the relative binding of penicillin N and adipoyl-6-APA to expandase in the ground state and subsequent intermediates and transition states for the expansion of these penicillins to DAOC and adipoyl-7-ADCA, respectively. Mutations at the aforementioned position of expandase will increase the expansion of adipoyl-7-ADCA, decrease the expansion of penicillin N and/or increase the relative ratio of adipoyl-7-ADCA to penicillin N expansion in a competitive scenario. This will result in an improved production process with an improved product/by-product ratio. Mutations are chosen in which the negative charge on position 109 is neutralized, or exchanged by a positively charged residue. Taking into regard the aspect that the mutations have to be accommodated by the structure without too many additional adaptations the following substitutions are preferred : D96N, D96Q, D96M, D96K, D96H (position 96 in *S.clavuligerus* corresponds with position 109 in aIPNS).

In order to improve adipoyl-6-APA as an isolated substrate it is necessary to improve  $V_{\max}$  and, in a context where the concentration of adipoyl-6-APA is non-saturating, to lower the  $K_m$ . This is not only the case when adipoyl-6-

APA is an isolated substrate but also when adipoyl-6-APA is a substrate in the presence of other penicillins, in the first place penicillin N but also isopenicillin N. The relative and absolute amounts of each penicillin expanded  
 5 depend on the ratio of the individual rates which can be broken down into an equation of the form:

$$\frac{V_{\text{adipoyl-6-APA}}}{V_{\text{penicillin N}}} = \frac{V_{\text{max}}^{\text{adipoyl-6-APA}} * K_m^{\text{penicillin N}} * [\text{adipoyl-6-APA}]}{V_{\text{max}}^{\text{penicillin N}} * K_m^{\text{adipoyl-6-APA}} * [\text{penicillin N}]}$$

10 where  $V_{\text{max}}$  corresponds to the maximum enzyme velocities,  $K_m$  is the Michaelis constant, and  $[\text{adipoyl-6-APA}]$  and  $[\text{penicillin N}]$  are the concentrations of adipoyl-6-APA and penicillin N respectively. Mutations at positions of the expandase listed below which result in an increase of the  
 15 ratio of  $V_{\text{max}}^{\text{adipoyl-6-APA}} : V_{\text{max}}^{\text{penicillin N}}$  are part of the invention. The specificity changes required can result from any single or multiple mutant that has values of  $V_{\text{max}}$  and/or  $K_m$  for either or both substrates altered in any way such as to increase the ratio of  $V_{\text{max}}^{\text{adipoyl-6-APA}} : V_{\text{max}}^{\text{penicillin N}}$  in vitro or the  
 20 relative yield of adipoyl-7-ADCA compared to DAOC from a adipic acid precursed fermentation of a strain of *P. chrysogenum* transformed with the mutant *cefE* gene.

Based on the proposal that the adipoyl carboxylgroup is  
 25 accommodated by positions which correspond to positions Arg87 and Ser183 in combination with our proposal that a negative charge in expandase at the position corresponding with aIPNS 109 is important for binding of the  $\alpha$  amino group, a number of positions can be derived from the  
 30 structural model which affect the specificity for the adipoyl side chain with respect to the  $\alpha$ -aminoadipoyl side chain of penicillin N.

Residues of *Streptomyces clavuligerus* expandase so  
 35 identified include, but are not restricted to:

Phenylalanine 152 (homologous to Threonine 180 of aIPNS),

Leucine 153 (homologous to Leucine 181 of aIPNS),  
Serine 187 (homologous to Serine 218),  
Arginine 266 (homologous to Asn 287 of aIPNS),  
Isoleucine 298 (homologous to Leucine 317 of aIPNS),  
5 Asparagine 301 (homologous to Glycine 320 of aIPNS),  
Tyrosine 302 (homologous to Leucine 321 of aIPNS),  
Valine 303 (homologous to Valine 322 of aIPNS).

10 Mutation of these residues individually or in combination  
will alter the relative binding of penicillin N and adipoyl-  
6-APA to expandase in the ground state and subsequent  
intermediates and transition states for the expansion of  
these penicillins to DAOC and phenylacetyl-desace-  
15 toxycephalosporin, respectively. Mutations at the  
aforementioned positions of expandase will increase the  
expansion of adipoyl-6-APA, decrease the expansion of  
penicillin N and/or increase the relative ratio of adipoyl-  
6-APA to penicillin expansion in a competitive scenario.

20 In *S.clavuligerus* position 109 is located at the start of a  
long loop which connects a  $\beta$ -strand and an  $\alpha$ -helix. This loop  
covers the  $\alpha$ -aminoadipoyl side chain. Modification of this  
loop adapts the specificity of expandase towards other  
substrates. Modification of this loop includes substitution  
25 of one or more aminoacids, insertions and deletions.

### Example 2

Shifting the substrate specificity of expandase by  
exchanging Asp96

30

Mutations at position 96 are chosen which change the  
negative charge at this position. The charge is neutralized,  
or exchanged by a positively charged residue. The following  
mutants are described: D96N, D96Q, D96M, D96K, and D96H.

35

a) General gene cloning and gene transformation procedures:

Common techniques used in gene cloning procedures are  
used in the present application. These techniques include  
polymerase chain reactions (PCR), synthetic oligonucleotide

synthesis, nucleotide sequence analysis, enzymatic ligation and restriction of DNA, *E. coli* vector subcloning, transformation, and transformant selection, isolation and purification of DNA. These techniques are all very well known in the art and adequately described in many references. See for example Sambrook et al., Molecular Cloning, a Laboratory Manual, Cold Spring Harbor, U.S.A. (1989), Innes et al., PCR protocols, a Guide to Methods and Applications, Academic Press (1990), and McPherson et al., PCR, a Practical Approach, IRL Press (1991).

General procedures used in transformation of filamentous fungi and transformant selection include preparation of fungal protoplasts, DNA transfer and protoplast regeneration conditions, transformant purification and characterization. These procedures are all known in the art and very well documented in: Finkelstein and Ball (eds.), Biotechnology of Filamentous Fungi, technology and products, Butterworth-Heinemann (1992); Bennett and Lasure (eds.), More Gene Manipulations in Fungi, Academic Press (1991); Turner, in: Pühler (ed.), Biotechnology, second completely revised edition, VCH (1992).

More specific applications of gene cloning and gene transformation technology to *Penicillium chrysogenum* are well documented in Bennett and Lasure (supra), Finkelstein and Ball (supra), and EP 0 357 119.

b) Construction of mutants on Asp96:

The expandase expression cassette pZEx, which contains the wild type *Streptomyces clavuligerus* expandase gene including the IPNS promoter and AT terminator, is constructed as described below. The *S. clavuligerus* expandase gene including the AT terminator is derived from plasmid pASEWA (described in WO 95/04149). pASEWA is cut with *Nde*I/ *Not*I, and the expandase-AT terminator fragment is isolated. The IPNS promoter is derived from *P. chrysogenum* chromosomal DNA in a PCR reaction using primers pcrA and pcrB (Table I), which are designed based on the IPNS promoter sequence (Smith et al. (1990), EMBO J. 9, p2743-

2750). The 0.9 kb PCR fragment is cut with *NdeI*/*NotI*, and the expandase- AT terminator fragment and the IPNS promoter fragment are ligated and inserted into the *NotI* site of pZErO (Invitrogen). Plasmid pZEx (Figure 2) is identified by restriction mapping.

The different expandase 96 mutants are constructed as follows: oligonucleotides (40-60 bases) are designed that cover the gene region between the *NdeI* site and the downstream *SacII* site in the expandase gene (see Figure 1).  
10 The oligonucleotides have the following characteristics:  
1. the *EcoNI* site is removed (oligonucleotides p2 and p8)  
2. the upstream *SacII* site is removed (oligonucleotides p4 and p11)  
3. the nucleotide sequence in p5 and p12 is varied in order  
15 to make the mutations at D96.

pZExD96N (Figure 3): oligonucleotides p1, p2, p3, p4, p5(N), p6, p7, p8, p9, p10, p11, and p12(N) (Table I) are annealed and ligated. The double stranded DNA molecules are amplified by PCR, using primers pcr1 and pcr12 (Table I). The  
20 resulting DNA fragment is cut with *NdeI* and *SacII*. pZEx is digested with these same enzymes, and mixed with the digested DNA fragment with the D96N mutation. After ligation, the plasmid DNA is cut with *EcoNI* and introduced into *E. coli* TOP10F. Plasmid pZExD96N is identified by  
25 restriction mapping using *EcoNI* and *SacII*, and the presence of the mutation at amino acid position 96 is confirmed by nucleotide sequence analysis.

pZEx-D96Q (Figure 4): this plasmid is constructed as described for pZExD96N, except that oligonucleotides p5(Q) and p12(Q) are used instead of p5(N) and p12(N),  
30 respectively (Table I).

pZEx-D96M (Figure 5): this plasmid is constructed as described for pZExD96N, except that oligonucleotides p5(M) and p12(M) are used instead of p5(N) and p12(N),  
35 respectively (Table I).

pZEx-D96K (Figure 6): this plasmid is constructed as described for pZExD96N, except that oligonucleotides p5(K) and p12(K) are used instead of p5(N) and p12(N), respectively (Table I).

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are purified by repeated cultivation on selective medium. Single stable colonies are used for further screening on the presence and expression of expandase by measuring the capacity of the transformants to produce cephalosporins.

5 Transformants are used to inoculate liquid medium as described in WO 95/04149, supplemented with 0.5-3 mg/ml sodium adipate as a side chain precursor for production tests. Filtrates of well grown cultures are analyzed by HPLC and NMR for production of adipoylcephalosporins and amino-

10 adipoylcephalosporins. Transformations with favourable adipoyl- over amino-adipoylcephalosporin production are selected.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

5

## (i) APPLICANT:

- (A) NAME: Gist-brocades
- (B) STREET: Wateringseweg 1
- (C) CITY: Delft
- (E) COUNTRY: Netherlands
- (F) POSTAL CODE (ZIP): 2311 XT

10

(ii) TITLE OF INVENTION: Improved Process for the Production of  
Adipoyl Cephalosporins

15

(iii) NUMBER OF SEQUENCES: 24

## (iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

20

## 25 (2) INFORMATION FOR SEQ ID NO: 1:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

30

(ii) MOLECULE TYPE: DNA (synthetic)

35

## (vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: pcr1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

40 GTTCGTAACA TATGGACACG ACGG

24

## (2) INFORMATION FOR SEQ ID NO: 2:

45

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 60 base pairs



- 21 -

- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: DNA (synthetic)

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: p2

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

TGCACCAAGA CGAGTTCCGC AGGTGTCTGA GGGACAAGGG CCTCTTCTAT CTGACGGACT  
60

15

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 60 base pairs
- 20 (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

25

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: p3

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

30

GCGGTCTGAC CGACACCGAG CTGAAGTCGG CCAAGGACAT CGTCATCGAC TTCTTCGAGC  
60

35 (2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 60 base pairs
- (B) TYPE: nucleic acid
- 40 (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

45 (vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: p4

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

ACGGCAGCGA GCGGAGAAG CGCGCCGTCA CCTCGCCCGT CCCCACCATG CGACGCGGCT  
60

5

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

10

(A) LENGTH: 60 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

15

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: p5(N)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

20

TCACCGGGCT GGAGTCGGAG AGCACCGCCC AGATCACCAA TACCGGCAGC TACTCCAACT  
60

25

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

30

(A) LENGTH: 60 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

35

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: p5(Q)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

40

TCACCGGGCT GGAGTCGGAG AGCACCGCCC AGATCACCAA TACCGGCAGC TACTCCCACT  
60

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 60 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5

(ii) MOLECULE TYPE: DNA (synthetic)

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: p5(M)

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

TCACCGGGCT GGAGTCGGAG AGCACCGCCC AGATCACCAA TACCGGCAGC TACTCCATGT  
60

15

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

20

- (A) LENGTH: 60 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: DNA (synthetic)

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: p5(K)

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

TCACCGGGCT GGAGTCGGAG AGCACCGCCC AGATCACCAA TACCGGCAGC TACTCCAAGT  
60

35

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

40

- (A) LENGTH: 60 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

45

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: p5(H)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

TCACCGGGCT GGAGTCGGAG AGCACCGCCC AGATCACCAA TACCGGCAGC TACTCCCACT  
60

5

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

- 10 (A) LENGTH: 40 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: DNA (synthetic)

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: p6

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

ACTCGATGTG CTACTCGATG GGCACCGCGG ACAACCTCTT  
40

25

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

- 30 (A) LENGTH: 40 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

35

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: p7

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

40

AGGCTGAAGG TGGGCACCGT CGTGTCCATA TGTTACGAAC  
40

45 (2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

- 25 -

- (A) LENGTH: 60 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5

(ii) MOLECULE TYPE: DNA (synthetic)

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: p8

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

CCCTTGTCCTC TCAGACACCT GCGGAACCTG TCTTGGTGCA GGCCCTGCTG GAGTTCGGCC  
60

15

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:

20

- (A) LENGTH: 60 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: DNA (synthetic)

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: p9

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

ATGTCCTTGG CCGACTTCAG CTCGGTGTGCG GTCAGACCGC AGTCCGTCAG ATAGAAGAGG  
60

35

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:

40

- (A) LENGTH: 60 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

45

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: p10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

ACGGGCGAGG TGACGGCGCG CTTCTCCGCC TCGCTGCCGT GCTCGAAGAA GTCGATGACG  
60

5

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:

10

(A) LENGTH: 60 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

15

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: p11

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

20

TTGGTGATCT GGGCGGTGCT CTCCGACTCC AGCCCGGTGA AGCCCGGTG CATGGTGGGG  
60

25

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:

30

(A) LENGTH: 60 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

35

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: p12(N)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

40

AAGAGGTTGT CCGCGGTGCC CATCGAGTAG CACATCGAGT AGTTGGAGTA GCTGCCGGTA  
60

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 60 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5

(ii) MOLECULE TYPE: DNA (synthetic)

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: p12(Q)

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

AAGAGGTTGT CCGCGGTGCC CATCGAGTAG CACATCGAGT ACTGGGAGTA GCTGCCGGTA  
60

15

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:

20

- (A) LENGTH: 60 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: DNA (synthetic)

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: p12(M)

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

AAGAGGTTGT CCGCGGTGCC CATCGAGTAG CACATCGAGT ACATGGAGTA GCTGCCGGTA  
60

35

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:

40

- (A) LENGTH: 60 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

45

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: p12(K)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

AAGAGGTTGT CCGCGGTGCC CATCGAGTAG CACATCGAGT ACTTGGAGTA GCTGCCCGTA  
60

5

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:

- 10 (A) LENGTH: 60 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: DNA (synthetic)

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: pl2(H)

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

AAGAGGTTGT CCGCGGTGCC CATCGAGTAG CACATCGAGT AGTGGGAGTA GCTGCCCGTA  
60

25

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:

- 30 (A) LENGTH: 60 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

35

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: pl

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

40

GTTCGTAACA TATGGACACG ACGGTGCCCA CCTTCAGCCT GGCCGAAGTC CAGCAGGGCC  
60

45 (2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:



- 29 -

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5

(ii) MOLECULE TYPE: DNA (synthetic)

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: pcr12

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

AAGAGGTTGT CCGCGGTGCC CATC

24

15

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:

20

- (A) LENGTH: 41 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: DNA (synthetic)

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: pcrA

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

CGTCTGGATC GCGGCCGCCT TATACTGGGC CTGCTGCATT G

41

35

(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:

40

- (A) LENGTH: 38 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

45

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: pcrB

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

CGTCTGGATC CATATGGGTG TCTAGAAAAA TAATGGTG

38

5

## Claims

- 5 1. A modified expandase gene encoding a mutant expandase which mutant expandase comprises:
- a) a substitution at one or more selected sites corresponding to a residue position selected from the group consisting of Aspartic acid 96, Phenylalanine 152, Leucine  
10 153, Serine 187, Arginine 266, Isoleucine 298, Asparagine 301, Tyrosine 302, Valine 303 in *S.clavuligerus* expandase,  
b) related to said wildtype expandase, an altered substrate specificity.
- 15 2. A modified expandase gene encoding a mutant expandase according to claim 1 which mutant expandase comprises one or more mutations selected from the group consisting of (a) D96N; (b) D96Q; (c) D96M; (d) D96K; (e) D96H.
- 20 3. An expression vector which comprises a modified expandase gene as defined in claim 1 or 2.
4. A microorganism host strain transformed with an  
25 expression vector as defined in claim 3.
5. An improved process for the preparation and recovery of 7-aminodesacetoxycephalosporanic acid (7-ADCA) by:
- 30 a) transforming a *Penicillium chrysogenum* strain with a modified expandase gene as defined in claim 1 or 2, under the transcriptional and translational regulation of fungal expression signals;  
b) fermenting said strain in a culture medium and adding to  
35 said culture medium adipic acid or a salt or ester thereof suitable to yield adipoyl-6-APA, which is expanded to form adipoyl-7-ADCA;  
c) recovering the adipoyl-7-ADCA from the fermentation broth;

- d) deacylating adipoyl-7-ADCA; and
- e) recovering the crystalline 7-ADCA.

6. A process according to claim 5, wherein step (e) is  
5 a filtration step.

7. A process according to claim 5 or 6, wherein step  
(c) is a filtration step, and by extracting the broth  
filtrate with an organic solvent immiscible with water at a  
10 pH of lower than about 4.5 and back-extracting the same with  
water at a pH between 4 and 10.

8. A process according to anyone of the claims 5, 6 or  
7 wherein the expandase gene is derived from *Streptomyces*  
15 *clavuligerus* or *Nocardia lactamdurans* or *Lysobacter*  
*lactamgenus*.

[illegible]

IPNS-E.nidulans	50	+	50	+	60	+	60	+	70	+	70	+	80	+	80	+	90	+
IPNS-S.clav	50	+	50	+	60	+	60	+	70	+	70	+	80	+	80	+	90	+
IPNS-S.anul	50	+	50	+	60	+	60	+	70	+	70	+	80	+	80	+	90	+
IPNS-S.lactam	50	+	50	+	60	+	60	+	70	+	70	+	80	+	80	+	90	+
IPNS-Flavob	50	+	50	+	60	+	60	+	70	+	70	+	80	+	80	+	90	+
IPNS-S.gris	50	+	50	+	60	+	60	+	70	+	70	+	80	+	80	+	90	+
IPNS-L.lactamgenus	50	+	50	+	60	+	60	+	70	+	70	+	80	+	80	+	90	+
IPNS_STRJU	50	+	50	+	60	+	60	+	70	+	70	+	80	+	80	+	90	+
IPNS-S.catt	50	+	50	+	60	+	60	+	70	+	70	+	80	+	80	+	90	+
DAOCS-S.clav	50	+	50	+	60	+	60	+	70	+	70	+	80	+	80	+	90	+
DACS-S.clav	50	+	50	+	60	+	60	+	70	+	70	+	80	+	80	+	90	+
DACS-S.lactam	50	+	50	+	60	+	60	+	70	+	70	+	80	+	80	+	90	+
DAOCS-Acrem	50	+	50	+	60	+	60	+	70	+	70	+	80	+	80	+	90	+
DACS-L.lactamgenus	50	+	50	+	60	+	60	+	70	+	70	+	80	+	80	+	90	+
DACS-L.lactamgenus_1	50	+	50	+	60	+	60	+	70	+	70	+	80	+	80	+	90	+
Consensus Identity	50	+	50	+	60	+	60	+	70	+	70	+	80	+	80	+	90	+



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		Page 4	
IPNS-E.nidulans	140 . . . + . . . 150 . . . + . . . 160 . . . + . . . 170 . . . + . . . 180 . . . + . . .	G A A L A L G	A
IPNS-S.clav	140 . . . + . . . 150 . . . + . . . 160 . . . + . . . 170 . . . + . . . 180 . . . + . . .	V G L A L L	
IPNS-S.anul	140 . . . + . . . 150 . . . + . . . 160 . . . + . . . 170 . . . + . . . 180 . . . + . . .	M L L V L M	
IPNS-S.lactam	140 . . . + . . . 150 . . . + . . . 160 . . . + . . . 170 . . . + . . . 180 . . . + . . .	V L L L G A L A L G	V
IPNS-Flavob	140 . . . + . . . 150 . . . + . . . 160 . . . + . . . 170 . . . + . . . 180 . . . + . . .	M L L V L M	
IPNS-S.gris	140 . . . + . . . 150 . . . + . . . 160 . . . + . . . 170 . . . + . . . 180 . . . + . . .	V V L L V L M	
IPNS-L.lactamgenus	140 . . . + . . . 150 . . . + . . . 160 . . . + . . . 170 . . . + . . . 180 . . . + . . .	V V L L V L M	
IPNS_STRJU	140 . . . + . . . 150 . . . + . . . 160 . . . + . . . 170 . . . + . . . 180 . . . + . . .	V V L L V L M	
IPNS-S.catt	140 . . . + . . . 150 . . . + . . . 160 . . . + . . . 170 . . . + . . . 180 . . . + . . .	V V L L V L M	
DAOCS-S.clav	120 . . . + . . . 130 . . . + . . . 140 . . . + . . . 150 . . . + . . . 160 . . . + . . . 170 . . . + . . . 180 . . . + . . .	V A A A V L	
DACS-S.clav	120 . . . + . . . 130 . . . + . . . 140 . . . + . . . 150 . . . + . . . 160 . . . + . . . 170 . . . + . . . 180 . . . + . . .	A A A A V L	
DACS-S.lactam	120 . . . + . . . 130 . . . + . . . 140 . . . + . . . 150 . . . + . . . 160 . . . + . . . 170 . . . + . . . 180 . . . + . . .	L A A A A L V L	
DAOCS-Acrem	120 . . . + . . . 130 . . . + . . . 140 . . . + . . . 150 . . . + . . . 160 . . . + . . . 170 . . . + . . . 180 . . . + . . .	L A A A A L V L	
DACS-L.lactamgenus	120 . . . + . . . 130 . . . + . . . 140 . . . + . . . 150 . . . + . . . 160 . . . + . . . 170 . . . + . . . 180 . . . + . . .	M G A A V A A V L	
DACS-L.lactamgenus_1	120 . . . + . . . 130 . . . + . . . 140 . . . + . . . 150 . . . + . . . 160 . . . + . . . 170 . . . + . . . 180 . . . + . . .	A L G A A V L	
Consensus Identity	120 . . . + . . . 130 . . . + . . . 140 . . . + . . . 150 . . . + . . . 160 . . . + . . . 170 . . . + . . . 180 . . . + . . .	L L G L A A A V L	



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IPNS-E.nidulans	180	190	200	210	220	230
IPNS-S.clav	180	190	200	210	220	230
IPNS-S.anul	180	190	200	210	220	230
IPNS-S.lactam	180	190	200	210	220	230
IPNS-Flavob	180	190	200	210	220	230
IPNS-S.gris	180	190	200	210	220	230
IPNS-L.lactamgenus	180	190	200	210	220	230
IPNS_STRJU	180	190	200	210	220	230
IPNS-S.calt	180	190	200	210	220	230
DACS-S.clav	180	190	200	210	220	230
DACS-S.clav	180	190	200	210	220	230
DACS-S.lactam	180	190	200	210	220	230
DACS-Acrem	180	190	200	210	220	230
DACS-L.lactamgenus	180	190	200	210	220	230
DACS-L.lactamgenus_1	180	190	200	210	220	230
Consensus	180	190	200	210	220	230
Identity	180	190	200	210	220	230

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IPNS-E.nidulans  
IPNS-S.clav  
IPNS-S.anul  
IPNS-S.lactam  
IPNS-Flavob  
IPNS-S.gris  
IPNS-L.lactamgenus  
IPNS\_STRJU  
IPNS-S.catt  
DAOCS-S.clav  
DACS-S.clav  
DACS-S.lactam  
DAOCS-Acrem  
DACS-L.lactamgenus  
DACS-L.lactamgenus\_1  
Consensus  
Identity

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IPNS-E.nidulans	370
IPNS-S.clav	.
IPNS-S.anul	.
IPNS-S.lactam	.
IPNS-Flavob	.
IPNS-S.gris	.
IPNS-L.lactamigenus	.
IPNS_STRJU	.
IPNS-S.call	.
DAOCS-S.clav	.
DACS-S.clav	.
DACS-S.lactam	.
DAOCS-Acrem	330
DACS-L.lactamigenus	IIA
DACS-L.lactamigenus_1	.
Consensus	.
Identity	.

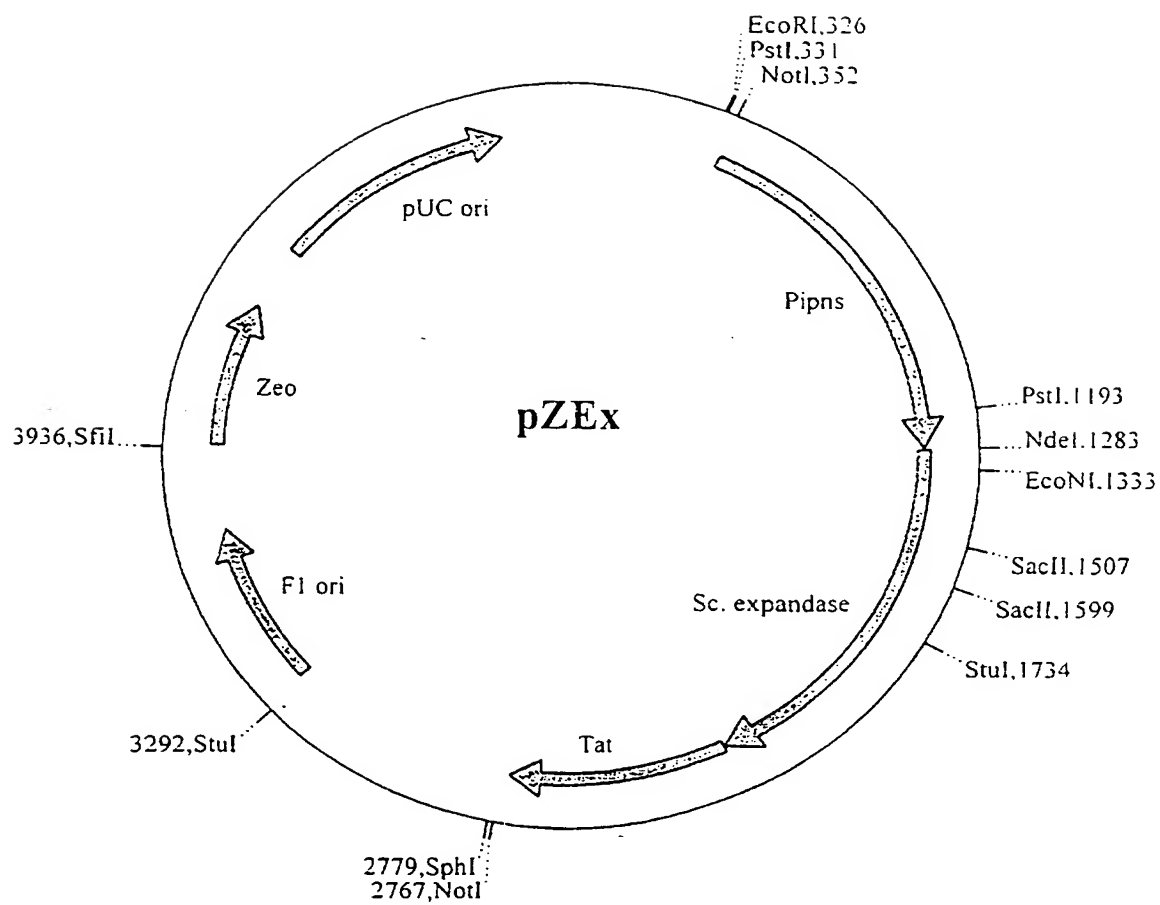


FIG. 2

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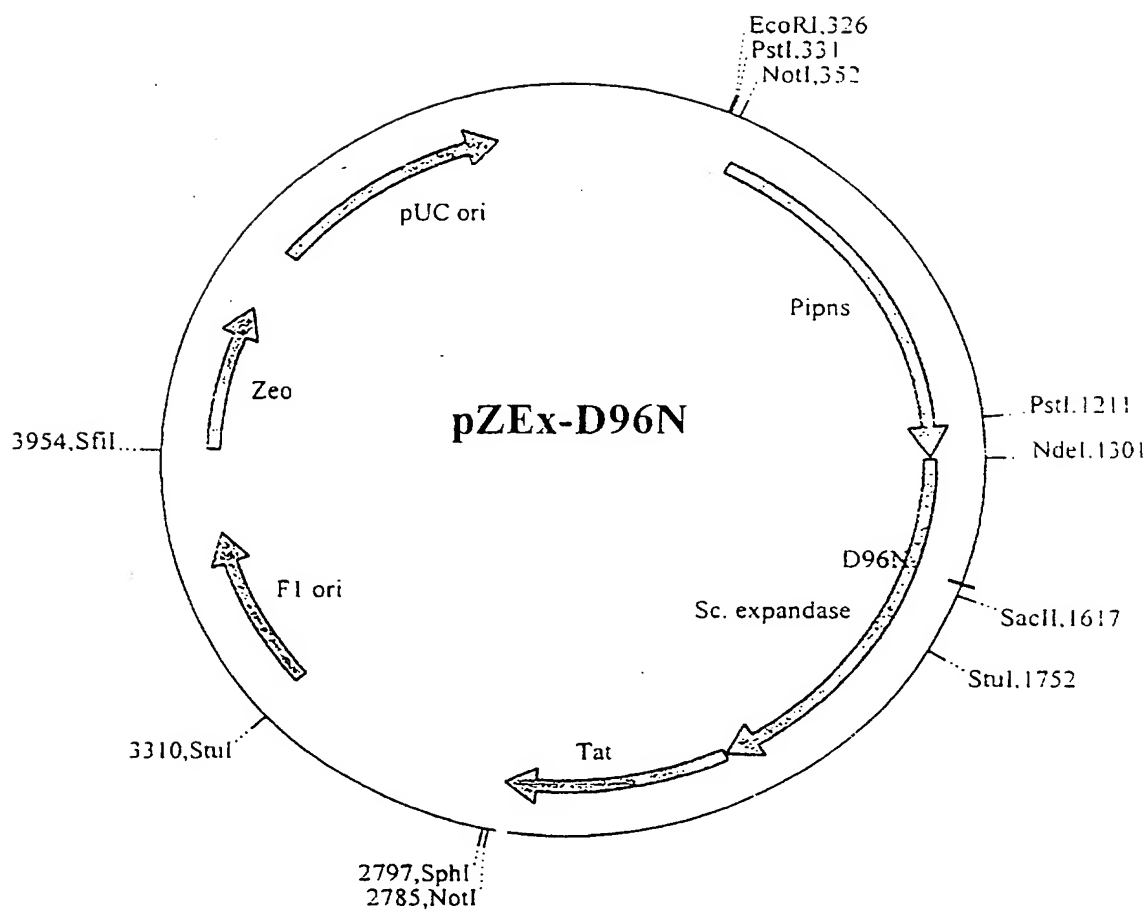


FIG. 3

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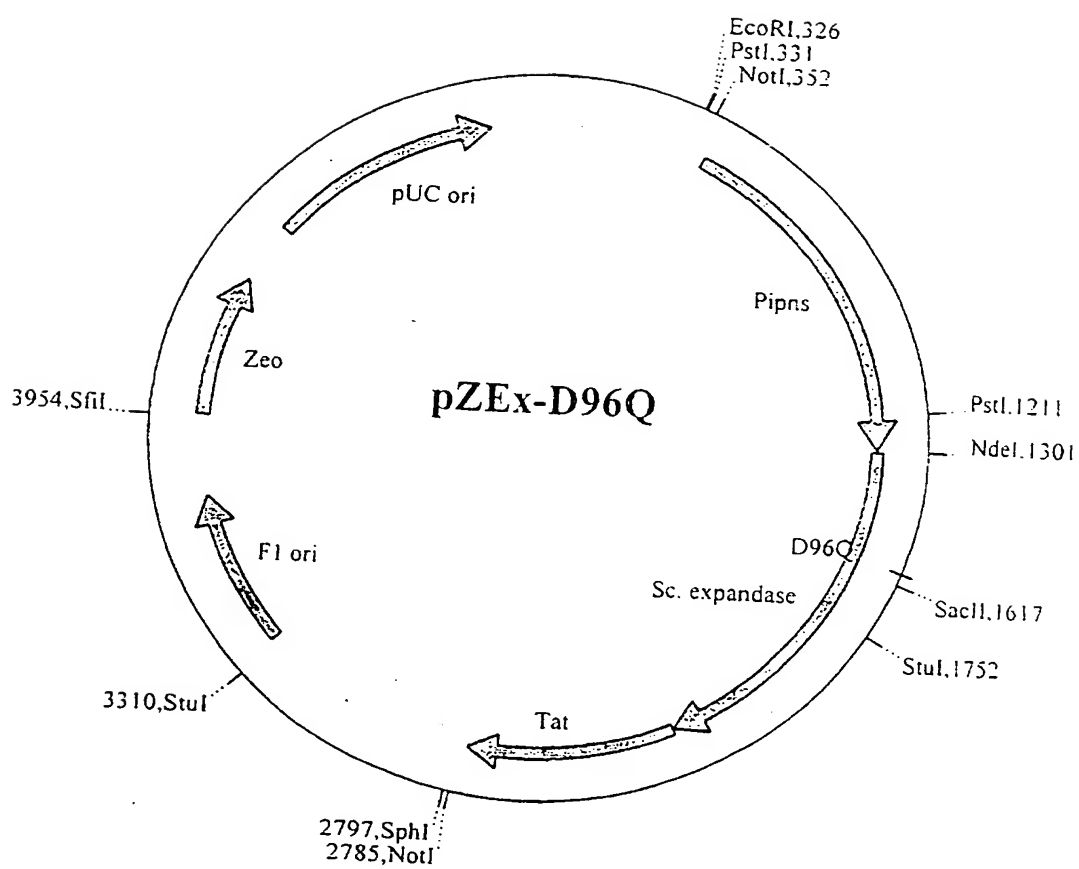


FIG. 4



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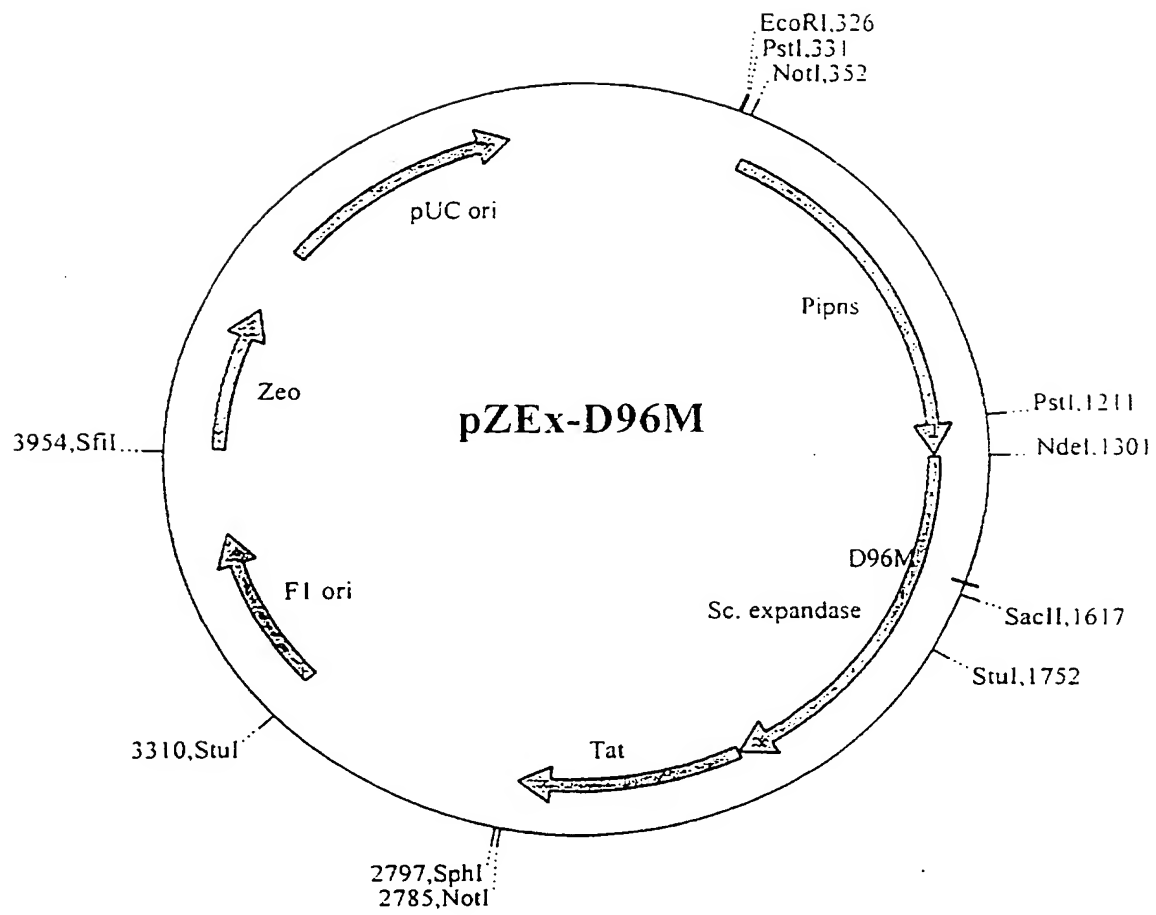


FIG. 5

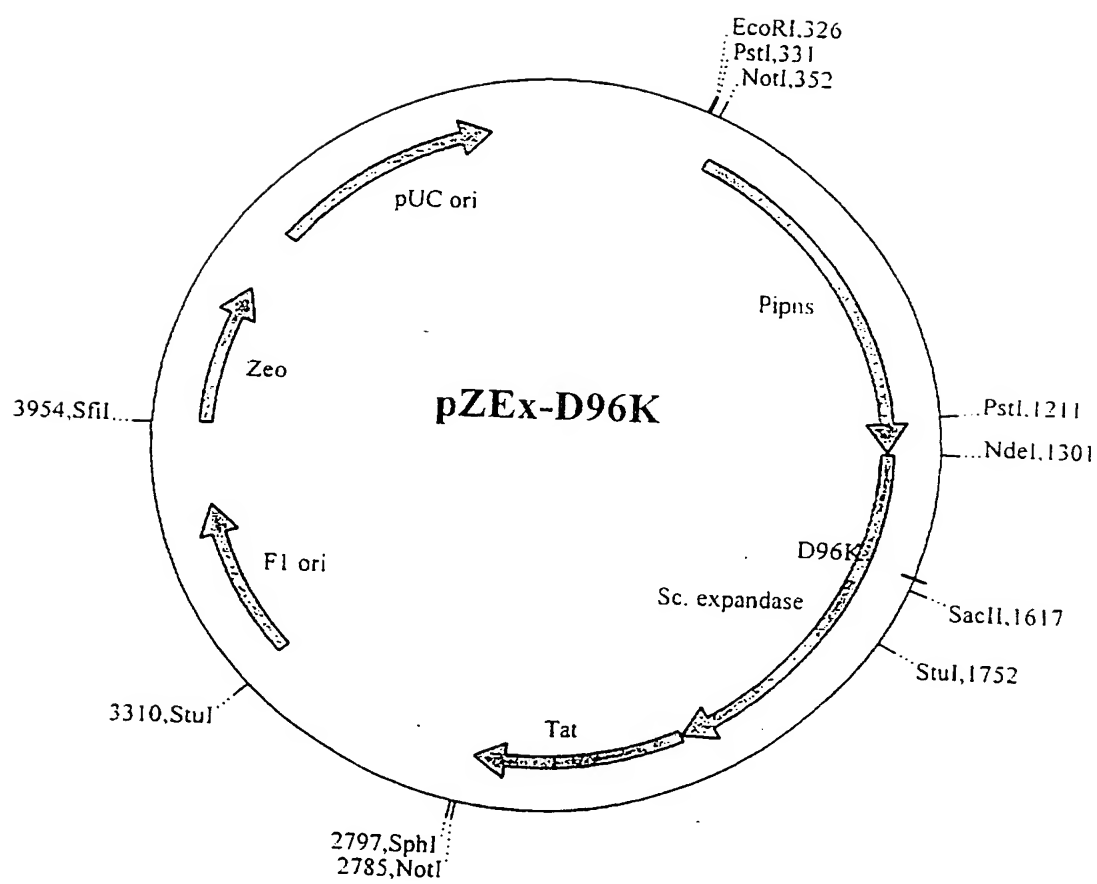


FIG. 6

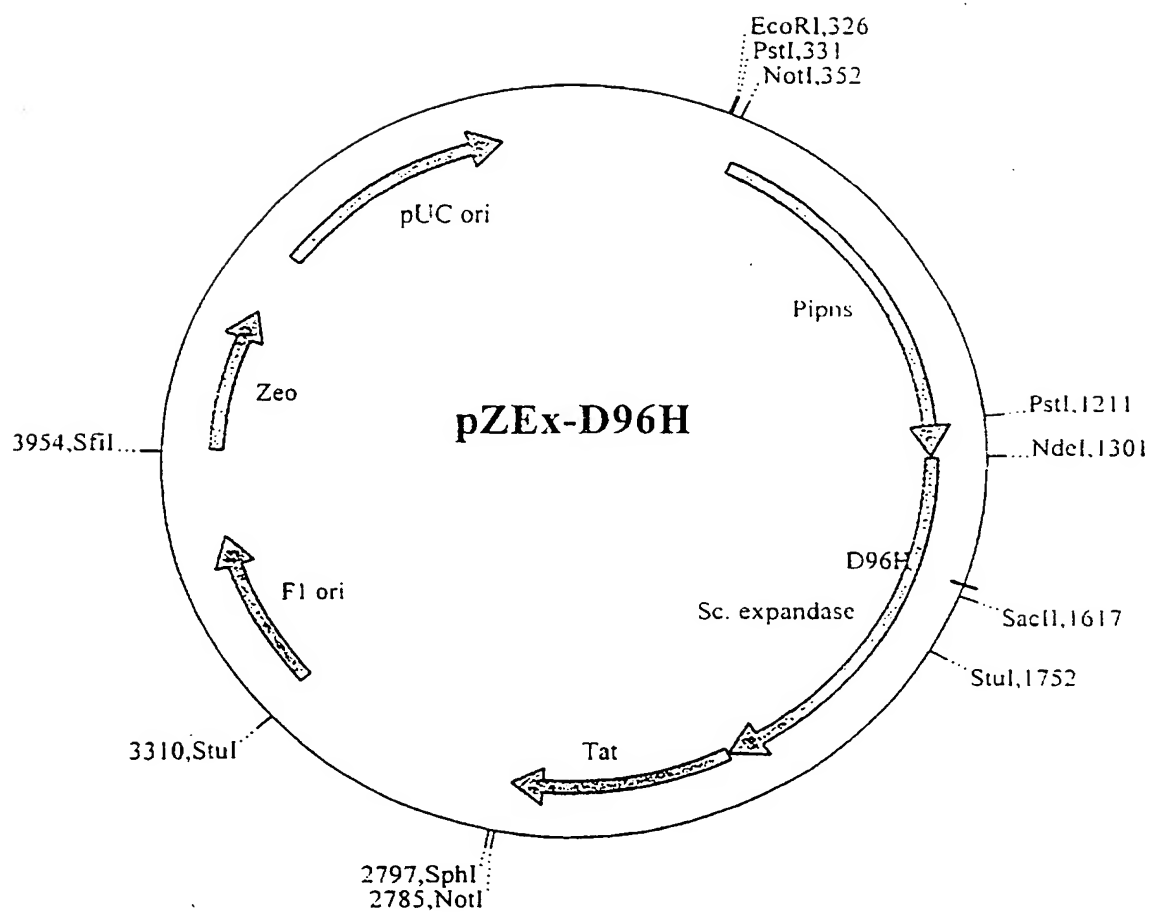


FIG. 7



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Information on patent family members

In International Application No

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